# Fusion of chromatophores from photosynthetic bacteria with a supported lipid layer: characterization of the electric units

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**Abstract Direct electrometric measurements of membrane potential changes are a valuable tool for study of vectorial transfer of electrons, protons, and ions. Commonly model membrane systems are created by fusion of lipid/protein vesicles with lipid-coated thin films. We characterized the electric units resulting from this process using chromatophores from the purple bacterium *Rhodobacter sphaeroides* and either a Mylar film or a planar modified gold electrode as support. Investigation of the shunting activity of the ionophore gramicidin on the flash-induced potential change demonstrates fusion of individual chromatophores to form independent 'blisters', which preserve an interior aqueous compartment. Under current–clamp conditions the photovoltage follows the change of the membrane potential of the individual blisters.** 

*Key words:* Photovoltage; Membrane potential; Membrane fusion; *Rhodobacter sphaeroides*

## 1. Introduction

The organization of the bioenergetic apparatus of living organisms in their native membrane system displays a fundamental asymmetry. Photosynthetic reaction centers (RC), bacteriorhodopsin, cytochrome $bc_1$, cytochrome *c* oxidase, and other integral membrane enzymes involved in bioenergetic reaction cycles are inserted in the membrane in a defined orientation. This structural asymmetry is the basis for creation of the electrochemical potential in the form of a proton gradient across the membrane, which drives formation of the universal energy carrier ATP by ATP synthases. Owing in particular to the intrinsic possibility of triggering the reaction sequence by light, photosynthesis has become a model system to study kinetics, energetics, and structure–function relationships – principles, which govern all biological reaction processes. In the RC of the purple bacterium *Rhodobacter sphaeroides* the complex reaction cycle of photosynthetic energy conversion starts with light-induced electron transfer from a primary electron donor (P) to a two-quinone ($Q_A$, $Q_B$) electron acceptor complex (see [1] for reviews). Due to the transmembrane organization of the cofactors electron transfer from P to $Q_A$ is electrogenic, i.e. it induces a change in the electric membrane potential, whereas electron transfer from $Q_A$ to $Q_B$ is not electrogenic. Coupled with the double reduction of the secondary quinone acceptor ($Q_B$) to quinol is the uptake of two protons from the cytoplasmic phase. This proton transfer to the $Q_B$ site is also electrogenic as are the reactions connected with oxidation of the quinol at the $bc_1$-complex.

Time-resolved photovoltage measurements are a very direct method to obtain information on kinetics and relative transmembrane distances of electrogenic charge transfer reactions. In photosynthetic systems intraprotein electron transfer as well as proton transfer has been studied by this technique (see [2,3] and references therein). A prerequisite for detection of a net electric signal is a preferential orientation of the macroscopic sample with respect to the electrodes. Flat membrane sheets can be oriented in an electric field. However, the useful time scale for photovoltage measurements is typically limited to some 100 ns due to ionic relaxations around such membrane sheets. To follow slower potential changes a well-sealed monolayer with low passive conductance is needed. A commonly applied technique is based on the fusion of photosynthetic vesicles or reconstituted proteoliposomes to a lipid-coated, thin collodion or Mylar film [4–6]. A slightly different technique makes use of a Teflon film as support to which a surface protein/lipid monolayer is adsorbed by slowly rising the level of the reservoir [7–10].

Although fusion 'monolayers' have been used since several decades, the physical architecture of the system and its implications for the electrical measurements have not been well characterized. Often the formation of the model membrane system is merely described as 'association' of vesicles with the lipid-coated support. The questions arise whether the layer formed on the support consists of a continuous structure or of separate units, what the size of these units is, and whether an interior aqueous phase is preserved. The latter point is of special interest as the integral membrane proteins often protrude out of the membrane and it is difficult to predict how they will accommodate with the support. Another point, which has not yet been addressed so far, concerns the impact of the type and the capacitance of the support through which the membrane potential change is coupled to the measuring circuit.

In this work we address these questions by investigating the effect on the flash-induced membrane potential of the type and the capacitance of the support, of addition of the iono-

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*Abbreviations:* RC, reaction center; P, primary electron donor; $Q_A$, $Q_B$, primary and secondary quinone acceptor; OM, *n*-octadecyl mercaptan; ODA, octadecylamine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; PMS, phenazine methosulfate

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phore gramicidin, and of venturicidin, an inhibitor of proton flow through ATPase. The results provide evidence that the formation of the model membrane involves a real fusion process of individual chromatophores forming separate blisters with an aqueous interior phase. Under current–clamp conditions the measured photovoltage is independent of the capacitance of the support and the kinetics follows the membrane potential of the individual blisters.

## 2. Materials and methods

### 2.1. Biological samples

Cells of *R. sphaeroides* R26 were grown anaerobically in Hutner media and stored at −20°C until use. Chromatophores were prepared by French-press treatment as described in [11] and purified by sucrose-gradient. Chromatophores were always freshly prepared.

### 2.2. Supports

Model membrane systems were created by fusion of the chromatophores to a lipid-impregnated solid support similarly as described [5,6]. As supports either a Mylar film (Goodfellow, thickness 2 μm) or a planar modified gold electrode was used. The area of the surface available for fusion was 0.75 $cm^2$ in both cases. For preparation of the gold electrode one surface of vigorously cleaned glass plates was covered successively with a 7 nm thick chromium layer and a 200 nm thick gold layer in a vacuum evaporation device (Edwards, E306; pressure $2–4\times10^{-6}$ mm Hg). The amount of metal deposited onto the glass plate was controlled by a quartz crystal microbalance. Freshly prepared gold electrodes were incubated for 3 h in a 1 mM ethanol/water (4/1, v/v) solution of *n*-octadecyl mercaptan (OM; Sigma). After autoassembly of the OM monolayer the electrodes were thoroughly rinsed with toluene, ethanol, and distilled water and used within 1 week.

### 2.3. Measuring cells

For the first set-up the film was clamped between two compartments of a polyethylene cell (volume ≈ 2 ml each). For the second set-up, only one compartment was used and the gold electrode was pressed against this compartment (Fig. 1). The compartment used in both set-ups was supplied with a window for light excitation. The lipid for impregnation of the support was a mixture of 1.2% (w/v) L-1,2-diphytanoyl-3-phosphatidylcholine (DPPC, Avanti Polar Lipids, Birmingham, AL, USA) and 0.025% (w/v) octadecylamine (ODA, Sigma) in *n*-decane (Merck). 20 μl of the lipid mixture were deposited on the support and allowed to dry for several hours to evaporate most of the solvent. The compartments were filled with electrolyte solution (10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 8, 100 mM KCl) and 50 μl of chromatophores ($OD_{860}\sim100$) were added. The suspension was stirred with small magnetic stirrers and fusion was induced by addition of 20 mM $CaCl_2$. After incubation at room temperature for about 1 h, the suspension was replaced by chromatophore-free and $Ca^{2+}$-free electrolyte solution.

### 2.4. Photovoltage measurements

Potential changes were detected via two electrodes made of Pt wire for the set-up with the film and two compartments or, for the second set-up, by one Pt electrode and the gold electrode, respectively. The two electrodes were connected to the input of an electrometer amplifier (input impedance $>10^{14}$ Ω, bandwidth DC-10 MHz) and signals were further amplified by a low noise preamplifier (Stanford Research Systems, model SR560, bandwidth 1 MHz). Single-shot signals were recorded on a digital storage oscilloscope (Tektronix, TDS 744A) on a fast time scale and in parallel on a data acquisition system of local design. The latter offered real-time averaging over a programmable number of digitization periods such as to yield a logarithmic time base from 1 μs to 10 s. A Faraday cage shielded the measuring cell and the electrometer amplifier. Excitation of the sample was by flashes from a Q-switched Nd-YAG laser (Quantel, France, FWHM 7 ns, $\lambda=532$ nm, energy ∼20 mJ/$cm^2$). Kinetic analysis was performed with a non-linear least square curve-fitting program (Microcal Origin). Simulations of the electrical equivalent circuit were performed with the program PSPICE (OrCAD).

## 3. Results and discussion

### 3.1. Comparison of different supports

Fig. 1 shows a schematic drawing of the gold/OM measuring cell and a simplified equivalent circuit of the ensemble. The equivalent circuit of the system with the Mylar film is essentially the same; however, for the film the capacitances describing the support, $C_c$ and $C_u$, are about 100 times smaller due to the larger thickness of the film compared to the OM/lipid layer. The biological membrane associated with the surface of the lipid-coated support is modeled by an ideal current source, a capacitance, and a resistance.

Typical photovoltage traces recorded with chromatophores from *R. sphaeroides* and with both types of lipid-coated supports, Mylar film or gold/OM electrode, are shown in Fig. 2. The amplitudes and kinetics of the photovoltage responses are comparable for both types of support and display the well-known features due to electrogenic events within the RC (see [2] for a review). Both flashes induce a fast, unresolved change of the potential becoming more negative by about 10 mV and arising from ultrafast transmembrane electron transfer from the P to the primary quinone acceptor ($Q_A$). On the second

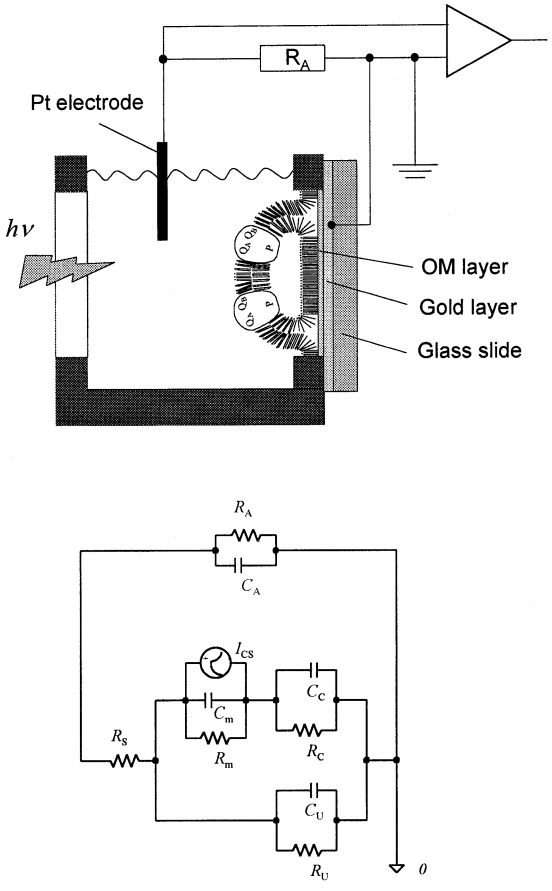


Fig. 1. Top: Schematic drawing of the experimental set-up. The half-sphere structure on the planar electrodes represents one of the blisters formed by fusion of chromatophores to the support (see text). Bottom: Simplified electric equivalent circuit of the electrode system. $I_{cs}$, ideal current source; $R_m$, $C_m$, resistance and capacitance of the membrane of fused chromatophores; $R_c$, $C_c$, and $R_u$, $C_u$ resistance and capacitance of the covered and uncovered area of the support, respectively; $R_s$, resistance of the electrolyte solution and electrode; $R_a$, $C_a$, external resistance and capacitance.

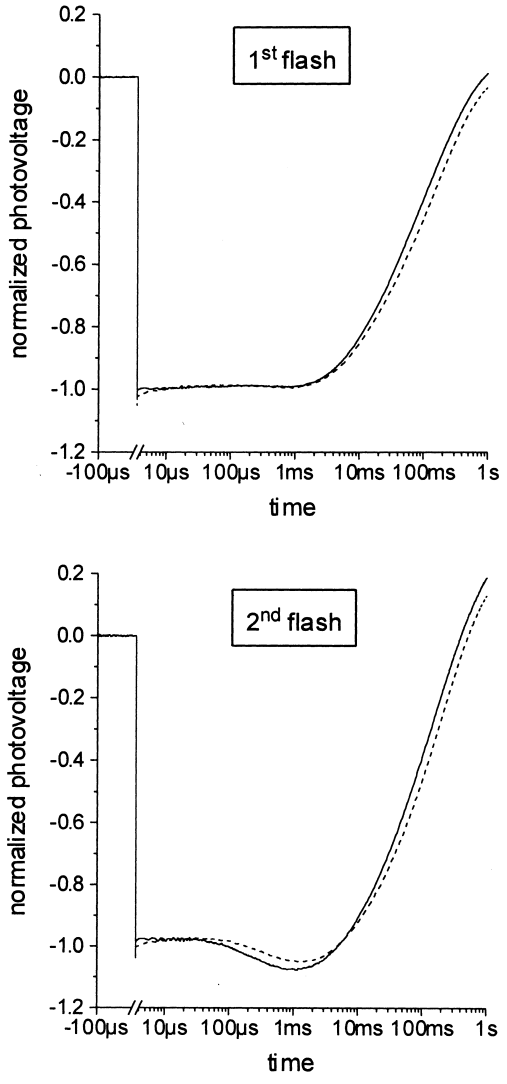


Fig. 2. Comparison of typical photovoltage traces obtained by flash excitation of chromatophores of *R. sphaeroides* fused to lipid-coated supports. (Dashed lines) gold/OM electrode, (solid lines) Mylar film. Response on the first (top) and on the second (bottom) flash. Time between flashes: 500 ms. Conditions: 10 mM HEPES (pH 8), 100 mM KCl, 1 mM ascorbate, 2 μM phenazine methosulfate (PMS). Traces were recorded on a logarithmic time scale and were normalized to equal amplitude at $t = 0$.

flash, an additional phase with the same polarity is clearly visible, which is due to electrogenic proton uptake upon double reduction of the secondary quinone acceptor ($Q_B$). The signals decay to the baseline within about 1 s after every flash.

The observation of a flash-induced potential difference with the characteristic features of charge transfer processes within the biological system allows two conclusions. First, the process of incubation of the chromatophore sample has led to an immobilization of the RC-containing vesicles on the lipid-coated support and, second, has led to a modification of the originally spherical symmetry of the vesicles creating an effective asymmetry of orientation. The polarity of the flash-induced potential changes indicates directly the direction of charge transfer with respect to the electrodes, i.e. the orientation of the majority of RCs. With chromatophores from *R. sphaeroides* fusion always resulted in an attachment with the donor side preferentially towards the lipid-coated support. In the original chromatophore vesicles the donor side of the RCs is towards the interior [12]. This allows to propose a mechanistic model for the process of association of chromatophores with the lipid-coated surface. The chromatophore vesicles open at the contact point with the lipid layer and a fusion process is initiated, which leads to loss of the symmetric shape of the vesicles and formation of blisters in which the former interior surface of the chromatophore membrane is now facing the planar lipid-coated support (see schematic presentation in Fig. 1). This proposed model of a real fusion process is confirmed by the observation of partial extraction of ubiquinones from the photosynthetic membranes (not shown; [13]) indicating that the hydrophobic quinones originally contained in the chromatophore membrane can diffuse into the lipid layer covering the support. A similar fusion process had been described for incorporation of quinones in supported phospholipid layers [14]. Electrostatic interactions seem to be important only during the fusion period as after this step $Ca^{2+}$ can be removed by exchange of the solution or addition of EDTA without effect on the signal. The independence of the observed photovoltage of the type of support also demonstrates that the essential interactions occur between the lipid layer and the vesicles.

If the proposed model of fusion is correct the orientation of the RCs on the support should be highly asymmetric as in chromatophores. This point was verified by comparing the photovoltage amplitudes induced by the first and the second flash after addition of the membrane-impermeable electron donor cytochrome *c*, which is able to reduce the photo-oxidized primary donor only in the minor fraction of RCs oriented with P facing the solution. In the presence of $Q_B$ inhibitors, where charge stabilization within the RC is limited to one turnover, this fraction will be blocked in the state $PQ_A^-$ after the first flash and will not contribute to the signal induced by the second flash. In contrast, the main fraction of RC with P inaccessible to cytochrome *c* (towards the support) will undergo charge recombination $P^+Q_A^- \rightarrow PQ_A$ within about 100 ms [2,15] and the second flash will induce again formation of $P^+Q_A^-$. The double flash experiment showed that addition

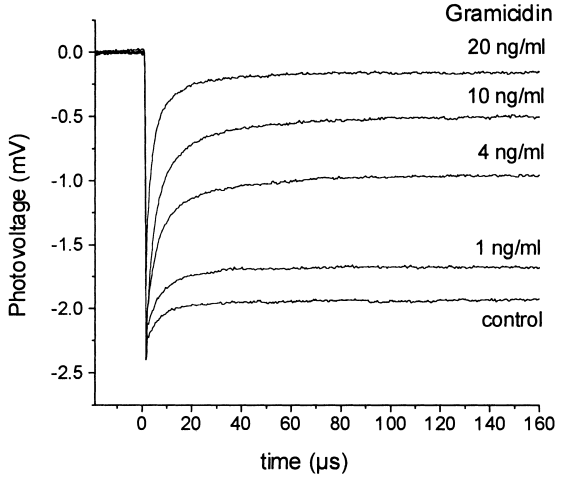


Fig. 3. Effect of gramicidin on the kinetics of flash-induced photovoltage changes of chromatophores of *R. sphaeroides* fused to a lipid-coated gold/OM electrode. Conditions as in Fig. 2.

of the external donor cytochrome *c* had only a very small effect (< 3%, not shown) on the amplitude induced by the second flash, the variation being within the accuracy of the measurement. This demonstrates that, at most, a very small amount of RC is oriented with the primary donor accessible to the exterior solution. It can therefore be concluded that the orientation of RCs in the model membrane is highly asymmetric and that the acceptor side of virtually all RCs is exposed to the external solution as it was in chromatophores.

### 3.2. Size of the structures formed by fusion: titration with gramicidin

To obtain information about the size of the structures formed by fusion of chromatophores we performed a titration with gramicidin, a well-characterized pore for monovalent cations (see [16] for a review). Fig. 3 shows the photovoltage kinetics in samples pre-incubated for 10 min with increasing concentrations of gramicidin. The effect of gramicidin is very pronounced and the same for both types of support (only traces obtained with the gold/OM system are shown in Fig. 3). With increasing concentration of the ionophore an increasing fraction of the photovoltage signal decays very rapidly within ~10 μs (the small phase of fast decay observed in this sample in the absence of gramicidin will be discussed below). The essentially biphasic behavior of the photovoltage kinetics is indicative for a statistic distribution of gramicidin leading to two populations of the electric units, one without active channel and one with one or more channels. The photovoltage signal being a superposition of contributions from all (Poisson-distributed) populations can be described by the equation [17]:

$$U(t) = U_0 \exp(-n) \exp(n \exp(-t/\tau))$$

where $n$ is the average number of gramicidin dimers per electric unit and $\tau$ is the discharge time of a unit by a single gramicidin dimer. Analysis of the traces in Fig. 3 with this time-dependence yielded good fits with $\tau = 11$ μs and $n$ ranging from 0.2 to 2.7 for increasing gramicidin concentration.

From the statistic effect of gramicidin it can be concluded that the fusion process resulted in formation of many independent electric units on the support. Furthermore, as the single channel conductance of gramicidin is known, the size of the corresponding units can be deduced from the discharge time $\tau$ of the membrane potential. According to the electric equivalent circuit (Fig. 1) gramicidin decreases the value of the resistance $R_m$ characterizing the conductance of the membrane of the units. The electrical discharge time in the presence of one gramicidin channel is given by $\tau = C_{unit}/G_1 = \langle C \rangle A_{unit}/G_1$, where $G_1$ is the single channel conductance of gramicidin, $\langle C \rangle$ is the specific capacitance of the membrane, and $C_{unit}$ and $A_{unit}$ are the capacitance and the membrane area of a single electric unit, respectively. With the time constant for the decay of the membrane potential in the presence of one gramicidin channel of $\tau = 11$ μs determined above, literature values for the single channel conductance of gramicidin A (for 100 mM KCl) of $G_1 = 12.3$ pS [18], and for the specific capacitance of the membrane $\langle C \rangle = 1.1$ μF cm$^{-2}$ [19] the membrane area and the capacitance of one electric unit can be estimated at $A_{unit} = 8 \times 10^{-11}$ cm$^2$ and $C_{unit} = 9 \times 10^{-17}$ F, respectively. There are no indications for a significant size distribution of units.

The mean diameter of chromatophores from *R. sphaeroides* has been determined by Packham et al. [19] as $d = 36$ nm, corresponding to a surface area of $A_{ch} = 4 \times 10^{-11}$ cm$^2$, and the capacitance of a single chromatophore as $C_{ch} = 3.8 \times 10^{-17}$ F. A somewhat larger mean diameter of chromatophores ($d = 60$ nm, corresponding to $A_{ch} \approx 11 \times 10^{-11}$ cm$^2$) has been determined from electron micrographs [12,20]. The values determined in the present work for the size and the capacitance of the electric units are thus within the range of values characterizing a single chromatophore vesicle. Furthermore, the decay time of the membrane potential in the presence of gramicidin determined in this work is very similar to the decay time of the electrochromic absorption changes obtained by gramicidin titration of chromatophores from *R. sphaeroides* in solution [21]. From these good agreements it can be concluded that the surface of the support is covered by independent blisters formed by fusion of single chromatophores. A flash-induced change of the membrane potential drives monovalent cations out of the fused vesicles through a gramicidin channel, showing that an internal aqueous phase is preserved.

Recently the structures formed by fusion of chromatophores to a collodion film have been studied by near-field scanning optical microscopy [22,23]. Based on trapping of dyes inside the chromatophores before fusion the correlation between topology and fluorescence images was taken as evidence for formation of isolated blisters with a definite aqueous interior. This is in agreement with the conclusions of the present work. On the other hand, Shinkarev et al. [22,23] observed heterogeneous populations of blisters with diameters of 0.2–10 μm and heights of 0.01–1 μm implying that 20–25 chromatophores fused together to create an average blister. This is in disagreement with the size of the electric units determined in situ by gramicidin titration in the present work. The discrepancy might be explained either by a fusogenic activity of the dye molecules [22,23] or a modification of the state of the monolayer by the treatment necessary for imaging, which involved air drying of the sample.

### 3.3. Relaxation of the photovoltage

In the presence of external, membrane-permeable electron donors charge separation within the RC is stable for seconds as evident e.g. from the presence of $Q_B^-$ 500 ms after the first flash (Fig. 2). However, photovoltage transients recorded under these conditions decay on a 100 ms time scale (Fig. 2). This evokes the question whether the capacitative coupling through the support and the external circuit disturbs the signal or whether the decay of the photovoltage is due to ionic leakage through the membrane of the blisters.

Simulations based on the equivalent circuit presented in Fig. 1 (bottom) show that the decay of the photovoltage is limited neither by the external circuit (as long as the external resistance is larger than 100 MΩ) nor by leakage through the lipid-coated support. The total capacitance of the supported layer $C_{tot} = C_u + C_m\ C_c/(C_m + C_c)$ can be easily measured. Typical values are $C_{tot} = 0.2$–$0.4$ μF for the gold/OM electrode system and $C_{tot} = 1.4$ nF for the Mylar film system. If the external circuit was limiting, the discharge times would be expected to be very different for the two types of support, which is not observed (with the specified input impedance of the electrometer amplifier of $R_a > 10^{14}$ Ω a discharge time ≫ 10 s is expected for both electrode systems). Similarly, the lower limit for the resistance of the lipid-coated support

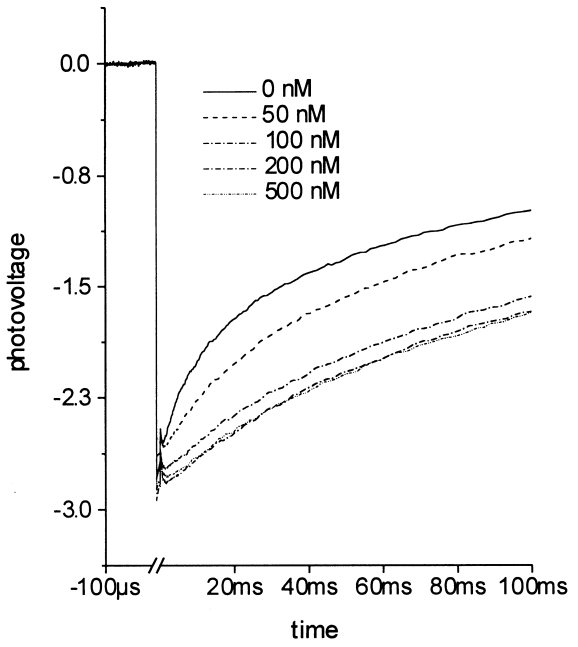


Fig. 4. Effect of venturicidin on the flash-induced photovoltage changes of chromatophores of *R. sphaeroides* fused to a lipid-coated Mylar film. Conditions as in Fig. 2.

($R_u$ $R_c/(R_u+R_c) \gg 100$ MΩ) as obtained from external measurements indicates a decay time caused by this pathway of >1s for both electrode systems. Therefore both pathways cannot explain the observed relaxation of the photovoltage.

This leaves leakage through the membrane of the blisters as explanation for the accelerated decay of the photovoltage. This pathway, like in the case of gramicidin, involves the effective conductance ($R_m^{-1}$) and capacitance ($C_m$) of the membrane of the blisters. The conductance could be either the intrinsic conductance of the membrane, an increased conductance (bad sealing) at the rims of the fusion regions, or the conductance of an ion channel or a specific ion translocating protein. It was noticed that the decay was often not monoexponential and accelerated at low pH or after addition of EDTA. As EDTA incubation is known to lead to removal of the $CF_1$ subcomplex of the ATPase [28], the possibility was considered that proton leakage by the $CF_0$ part contributes to the decay of the membrane potential in the individual blisters. To investigate this point we performed a titration with venturicidin, a specific inhibitor of proton flow through $CF_0$ [24]. The relaxation of the photovoltage was clearly slowed by increasing concentrations of venturicidin (Fig. 4). A kinetic analysis showed that the relaxation is biphasic and that venturicidin slows the faster component, which is responsible for about 30% of the decay. Its time constant increases from 20 ms in the absence of venturicidin to 70 ms at 100 nM venturicidin. Higher concentrations did not lead to a further slow down. The slower phase of the decay (~500 ms) was essentially unaffected by the inhibitor. A very similar effect of venturicidin on the membrane potential of chromatophores in solution has been reported by Saphon et al. [25]. These observations indicate that ATPase can contribute to the conductance in part of the fused vesicles. This conductance is not necessarily related to removal of the coupling factor $CF_1$, as it is known that ATPase is leaky in the absence of nucleotides performing uncoupled $H^+$ transport ('proton slip'; [26]). The about 2000 times slower decay time compared to gramicidin gives an estimation of ~5 fS for the conductance of this pathway in agreement with the time-averaged conductance determined for single channels of the $CF_0$–$CF_1$ enzyme complex [27]. The main phase of relaxation, independent of the presence of venturicidin, is more than one order of magnitude slower and is attributed to residual permeability of the fused membranes. Occasionally a small phase of very fast relaxation of the photovoltage is observed (see Fig. 3, control, for an example). This phase, the kinetics of which depend on the ionic strength, is tentatively assigned to bad sealing of part of the blisters.

In summary, the characterization of a supported membrane system formed by fusion of vesicles presented in this work shows that under current–clamp conditions the photovoltage kinetics is directly related to changes of the membrane potential of the individual vesicles. The identification of the individual blisters as the basic electric units is essential to understand the electrical behavior of the system, which can not be modeled as one homogeneous interfacial layer.

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